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ORIGINAL ARTICLE

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ABSTRACT

Objectives: We hypothesized that LOX-1 polymorphism may impact on inflammation and cardiovascular risk by modulating systemic resistin expression.

Design and Methods: 276 men were randomly selected from a population-based cohort. Metabolic and inflammatory markers were evaluated at baseline and after 6-years follow-up, *OLR1* (loxin) IVS4-14 A>G polymorphism was assessed.

Results: Mean plasma resistin and nitrotyrosine values were significantly higher, and TAS was significantly lowered in homozygous for the G allele. The G allele was significantly and directly associated with resistin and nitrotyrosine values.

Conclusion: Enhanced oxidized-LDL uptake by LOX-1 G-allele carriers is associated with increased pro-oxidant status and resistin levels, suggesting a major uptake of ox-LDL by macrophages, smooth muscle cells, and monocytes.

INTRODUCTION

Low-density lipoprotein (LDL) accumulation and oxidative modification in the subendothelium has been found to promote monocyte and lymphocyte recruitment, triggering atherosclerosis. LOX-1 is a scavenger receptor which was first identified in endothelial cells and subsequently found to be expressed by macrophages and smooth muscle cells (SMC) (1). Upon ligand binding, LOX-1 activation leads to intracellular signaling which may cause the onset of cardiovascular events or accelerate the development of atherosclerosis: enhanced secretion of enzymes, such as matrix metalloproteases by endothelial cells, increased superoxide production leading to reduced nitric oxide levels, and transcription factor NF-kB activation (2).

LOX-1 is a type II membrane protein belonging to the C-type lectin family and it is composed of four domains: an N-terminal cytoplasmatic domain, a single transmembrane domain, a connecting neck domain, and a lectin-like domain at the C-terminus. The conserved among species C-terminal residues are essential for LDL binding. LOX-1 is a disulfide-linked homodimer and dimerization is required for its ligand-binding activity (3). Atheroma-derived cells over-express LOX-1 in human lesions (3). Protease can cleave the receptor at the juxtamembrane region and the soluble form of LOX-1 is released (4).

LOX-1 is encoded by a single gene, named *OLR1*, mapped on chromosome 12p12.3-13.1. Several polymorphisms within the *OLR1* gene were reported to be associated with coronary atherosclerosis and acute myocardial infarction (5). A new functioning splicing of the *OLR1* gene lacks exon 5 and it is named *loxin*. Levels of loxin mRNA and protein expression were negatively associated with the incidence of myocardial infarction in humans. Loxin lacks the ligand binding site, but interacts with the full-length LOX-1 receptors by blocking their cellular expression, ox-LDL binding activity, and uptake (5).

The *OLR1* linkage disequilibrium (LD)1 polymorphism regulates the expression of loxin mRNA. Macrophages of subjects with the “no risk” polymorphism have higher levels of mRNA as well as protein expression than macrophages of subjects carrying the risk haplotype.

Macrophages are the main source of resistin in humans. Resistin belongs to the family of resistin-like molecules known as “found in inflammatory zone” (FIZZ), and it has been implicated in the regulation of inflammation through NF-kB pathway activation (6).

The IVS4-14 A>G polymorphism of human *OLR1* gene influences the transcription of the two isoforms LOX-1/LOXIN whose ratio could allow the identification of subjects who are at risk of cardiovascular disease. Subjects carrying the mutant G allele express less loxin than those with the wild type A allele.

The aim of the present work was to investigate how this polymorphism could influence serological markers of lipid metabolism, inflammation and cytokine production after LOX-1 activation.

METHODS

A cohort of 1658 subjects aged 45-64 years and living in the province of Asti (north-western Italy) was enrolled in a metabolic survey. The subjects were evaluated at the beginning of the study (2001-2003) and then in 2008, after a mean follow-up time of 6.1±0.34 years. Out of them, 300 men were identified by using the simple random sampling procedure and submitted to the measurement of serum resistin, nitrotyrosine (NT) and total antioxidant status (TAS) values. The stored blood samples of 276/300 cases were available for the determination of the IVS4-14 A>G polymorphism of human *OLR1 gene.*

Characteristics of subjects who were included in the study and those who were not, did not significantly differ (data not shown) and were similar to these of the whole cohort. The following have been measured: weight, height, waist circumference, blood pressure, values of fasting glucose, insulin, total cholesterol, HDL-cholesterol (HDL-Chol), triglycerides (Tg), high-sensitivity C-reactive protein (hs-CRP), nitrotyrosine total antioxidant status, resistin levels and IVS4-14 A>G polymorphism of human *OLR1* gene (loxin).

We have studied only men in order to avoid any possible bias due to sex differences in serum resistin levels, which have been demonstrated to be higher in women.

Informed consent was signed from all participants, all procedures were in accordance with the Declaration of Helsinki and this study was approved by the local Ethical Committee.

Diabetes and impaired fasting glucose (IFG) were diagnosed according to published recommendations (7) Hypertension and cardiovascular disease were diagnosed in line with the National Cholesterol Education Program (Adult Treatment Panel III) (NCEP-ATP III) criteria (8).

Genotyping for IVS4-14 A>G polymorphism of human gene OLR1 was performed with the loxin test (Technogenetics, Sesto San Giovanni, Italy) through polymerase chain reaction followed by electrophoresis on agarose gel.

Allele frequencies were calculated by gene counting. Values were expressed as mean ± SD, unless otherwise specified.

ANOVA and the chi-square test were used to compare means for continuous variables or frequencies for discrete variables, respectively.

Multiple regression analyses were used to evaluate the associations among values of NT, resistin, TAS, and presence of G allele, after adjustment for age, BMI, smoking habits, baseline diabetes, hypertension and coronary artery disease.

RESULTS

We considered three groups of subjects according to their IVS4-14 A>G genotype. The IVS4-14 A>G genotypic distribution in this population was in Hardy-Weinberg equilibrium. The normal homozygous genotype (AA) had a frequency of 40.9 %, the mutant homozygous genotype (GG) had a frequency of 27.5 % and the heterozygous genotype (AG) had a frequency of 31.5%. The G allele frequency was 0.43. Physical activity level and dietary intakes did not differ among genotype groups.

Table 1 shows the distribution of mean baseline resistin, NT and TAS levels among the IVS4-14 A>G genotypes. The mean resistin and NT values were significantly higher, while TAS was significantly lower in homozygous G allele carriers.

In a multiple regression model, resistin and NT values are significantly and directly associated with G allele, while TAS is inversely associated. This association kept significant after adjustment for age, BMI, smoking habits, presence of diabetes, hypertension and coronary artery disease at baseline (Table 2).

DISCUSSION

Our data suggest a significant association between loxin polymorphism and plasma resistin, NT and TAS. No association was found with markers of lipid metabolism. Resistin belongs to the family of resistin-like molecules (RELMs), and it also known as "found in inflammatory zone" (FIZZ). In humans, macrophages and adipose tissue are the main source of resistin (6). In human macrophages, resistin enhances secretion of pro-inflammatory cytokines, TNF-α and IL-12, and is able to induce the nuclear translocation of NF-κB transcription factor (6) which plays a key role in regulating the expression of proinflammatory genes including those for vascular adhesion molecules and MCP-1 (9). Therefore, resistin may mediate the association between increased LOX-1 stimulation and the NF-κB-triggered pro-inflammatory and pro-oxidative states which predispose to the development of atherosclerosis (2). The finding of higher circulating resistin levels in subjects carrying the G allele suggests a major uptake of ox-LDL by LOX-1-expressing macrophages, smooth muscle cells, and monocytes~~,~~. Altogether, these data suggest that in G allele carriers a higher amount of ox-LDL is up-taken by mononuclear cells, reinforcing the proinflammatory activation of macrophages and the release of resistin.

Our data also suggest the peroxynitrite production may mediate LOX-1-mediated pro-inflammatory activity. Ox-LDL binding to LOX-1 enhances reactive oxygen species (ROS) through the activation of a membrane-bound NADPH oxidase (10). Subjects carrying the variant G allele have more LOX-1 which are not blocked by loxin than those with the A allele. More molecules of LOX-1 on the surface of endothelial cells mediate the internalization of Ox-LDL and cause the activation of NADPH-oxidase. Consistent with this model, TAS was significantly lower in carriers of the G allele as a consequence of antioxidant~~s~~ consumption to buffer a rise in ROS levels.

hs-CRP values kept quite similar among the three groups despite the differences in resistin levels. hs-CRP is unlikely to be influenced by such low values of resistin. Resistin might influence the levels of other cytokines at higher concentrations. Nonetheless, resistin, even at very low values, might influence the local environment. Differences in acetylsalicylic acid use might be a reason for variation in hs-CRP values from baseline to follow-up. Similarly, differences in the proportion of patients treated with anti-hypertensive drugs or differences in the use of insulin sensitizers or insulin secretagogues drugs might justify differences in the variable values from baseline to follow-up among genotype groups.

We were unable to find a genotype-dependent increase in coronary artery disease or diabetes in our cohort of subjects over the entire period of follow-up, since our study did not have sufficient statistical power to detect differences between the groups owing to the low number of incident

events. Nevertheless, we provided evidence for an involvement of loxin polymorphism in the LOX-1-regulated intracellular signaling towards a pro-inflammatory state in the endothelium.

In conclusion, our results suggest that loxin polymorphism is likely to play a role in the early stages of the atherosclerotic process and that increased resistin secretion characterizes the pro-oxidant state associated with enhanced ox-LDL uptake by LOX-1. This is a population-based study. Replication in other centers/populations is likely to be necessary, and future prospective studies are needed to determine whether resistin plays a role in the development of clinical atherosclerosis and if resistin antagonism may reduce the increased cardiovascular risk of LOX-1 mutated allele.

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Author Disclosure Statement
No competing financial interests exist

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Table 1 Distribution of mean baseline resistin, NT and TAS levels among the IVS4-14 A>G genotypes.

	GG	**AG**	**AA**	**P**
Number	76	87	113	
Resistin (ng/ml)	3.6±1.8	3.5±2.0	2.9±1.3	0.02
Nitrotyrosine (nmol/ml)	6.6 (12.8)	5.1 (6.6)	4.1 (6.3)	<0.001
Total Antioxidant Status (mmol/l)	0.36±0.26	0.44±0.29	0.67±0.26	<0.001

Table 2 Multiple regression analysis

	AG	**GG**	**AG**	**GG**
	Crude		**Adjusted***	
	β; 95%CI, p	β; 95%CI, p	β; 95%CI, p	β; 95%CI, p
Resistin (ng/ml)	0.59; 0.10 1.08, 0.02	0.63; 0.12 1.14, 0.02	0.62; 0.13 1.11, 0.01	0.59; 0.08 1.10, 0.02
Nitrotyrosine (nmol/ml)	0.51; 0.10 0.92, 0.01	1.03; 0.60 1.46, <0.01	0.51; 0.10 0.92, 0.02	1.02; 0.59 1.45, <0.01
Total Antioxidant Status (mmol/l)	-0.23; -0.31 – 0.15,<0.01	-0.31; -0.39 –0.23, <0.01	-0.23; -0.31 –0.15, <0.01	-0.30; -0.38 –0.22, <0.01

* after adjustment for age, BMI, smoking habits, baseline diabetes, hypertension and coronary artery disease